

PATENT COOPERATION TREATY

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Article 36 and Rule 70)

Applicant's or agent's file reference NEB-150-PCT	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US99/22776	International filing date (day/month/year) 30 September 1999 (30.09.1999)	Priority date (day/month/year) 30 September 1998 (30.09.1998)
International Patent Classification (IPC) or national classification and IPC IPC(7): C12N 9/64, 15/57, 15/62, 15/70, 15/79, 15/866; C12Q 1/37; C07K 16/40; A61K 38/48, 39/395 and US Cl.: 435/226, 23, 69.1, 69.7, 252.3, 252.33, 320.1; 536/23.2, 23.7; 530/387.1; 514/1; 510/305		
Applicant NEW ENGLAND BIOLABS, INC.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets, including this cover sheet.

This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of 0 sheets.

3. This report contains indications relating to the following items:

- I Basis of the report
- II Priority
- III Non-establishment of report with regard to novelty, inventive step and industrial applicability
- IV Lack of unity of invention
- V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI Certain documents cited
- VII Certain defects in the international application
- VIII Certain observations on the international application

Date of submission of the demand 02 February 2000 (02.02.2000)	Date of completion of this report 11 July 2001
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer Ponnathapura Achutamurthy Telephone No. 703.308.0196

Forn PCT/IPEA/409 (cover sheet)(July 1998)

I. Basis of the report1. With regard to the **elements** of the international application:*

the international application as originally filed.

the description:

pages 1-22 _____ as originally filed

pages _____, filed with the demand

pages _____, filed with the letter of _____

the claims:

pages 23 and 24 _____ as originally filed

pages _____, as amended (together with any statement) under Article 19

pages _____, filed with the demand

pages _____, filed with the letter of _____

the drawings:

pages 1-11 _____, as originally filed

pages _____, filed with the demand

pages _____, filed with the letter of _____

the sequence listing part of the description:

pages 1-6 _____, as originally filed

pages _____, filed with the demand

pages _____, filed with the letter of _____

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item. These elements were available or furnished to this Authority in the following language _____ which is:

the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

the language of publication of the international application (under Rule 48.3(b)).

the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

contained in the international application in printed form.

filed together with the international application in computer readable form.

furnished subsequently to this Authority in written form.

furnished subsequently to this Authority in computer readable form.

The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

4. The amendments have resulted in the cancellation of:

the description, pages _____

the claims, Nos. _____

the drawings, sheets/fig _____

5. This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

* Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).

** Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US99/22776

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**1. STATEMENT**

Novelty (N)	Claims <u>7 and 11</u>	YES
	Claims <u>1-6 and 8-10</u>	NO
Inventive Step (IS)	Claims <u>7 and 11</u>	YES
	Claims <u>1-6 and 8-10</u>	NO
Industrial Applicability (IA)	Claims <u>1-11</u>	YES
	Claims _____	NO

2. CITATIONS AND EXPLANATIONS (Rule 70.7)

Claims 1-6 and 9 lack novelty under PCT Article 33(2) as being anticipated by Muir et al. who disclose, in June 1998, a method - for fusing a recombinant expressed first, target, polypeptide encoded by a nucleic acid sequence comprised within the plasmid pCYB2, see Figure 2 and the text at pages 6706-6707, and having a carboxyl-terminal thioester - generated by thiophenol-induced cleavage of an intein having an amino-terminal cysteine fused to the target polypeptide - with a second, target, peptide having an amino-terminal cysteine. Muir et al. disclose that the fusion of the target polypeptide to the target peptide is accomplished *in vitro* by chemical ligation wherein a nucleophilic attack by the amino-terminal cysteine of the target peptide upon the carboxyl-terminal phenyl thioester of the target polypeptide results in formation of peptide bond uniting the target polypeptide amino-proximal to the target peptide, meeting the limitations of claims 1-6 and 9. Muir et al. further disclose, page 6707, that they screened other thiol reagents in addition to thiophenol to induce cleavage of the intein, encoded by the plasmid pCYB2, comprised within a precursor protein including the fused first, target, polypeptide.

Claims 9 and 10 lack novelty under PCT Article 33(2) as being anticipated by Chong et al. who in 1997 disclose a method of generating a reactive thioester, see Figure 1 at page 273, at the carboxyl terminus of a target protein by contacting a precursor protein comprising a target protein and an intein with a thiol reagent such as dithiothreitol or beta-mercaptoethanol, meeting limitations of claim 9 and the preamble and clauses (a) and (b) of claim 10. Chong et al. further disclose, see Figure 3 at page 275, a process of determining whether or not a cleavage event occurred after contacting a thiol reagent with a precursor protein comprising an intein and a target polypeptide, thus also meeting the limitations of clause (c) of claim 10.

Claim 8 lacks novelty under PCT Article 33(2) as being anticipated by Telenti et al. who in 1997 disclose a modified intein that comprises a mutant *Mycobacterium xenopi* GyrA intein capable, see results depicted in Table 1 with the C114R mutant in the "MIEP" expression construct at page 6380, of thiol reagent-induced cleavage producing a thioester at the carboxyl-terminus of an intein comprised within a precursor protein including a fused polypeptide which inherently may serve as target protein upon cleavage, meeting limitations of claim 8.

Claims 7 and 11 meet the criteria set out in PCT Article 33(2)-(4), because the prior art does not teach or fairly suggest the subject matter they describe: a cyclic protein of claim 7 produced by an *in vitro* chemical ligation between a carboxyl-terminal thioester of a target protein and an amino-proximal cysteine of another region of the target protein or a method of claim 11 where ligation of a cytokine target peptide serves in determining the occurrence or non-occurrence of a splicing or cleavage event. Claims 1-6 and 8-10 also meet the criteria set out in PCT Article 33(4) because they describe subject matter both practicable and useful.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US99/22776

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

Claim 11 is objected to under PCT Rule 66.2(a)(iii) as containing the following defect(s) in the form or contents thereof: the word "terminal" is misspelled at line 4 of claim 11.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/22776

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C07K 19/00; C12N 9/10, 15/62; C12P 21/02, 21/04
US CL :435/68.1, 69.7; 513/2; 530/402, 408; 536/23.4

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/68.1, 69.7; 513/2; 530/402, 408; 536/23.4

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	US 5,834,247 A (COMB et al) 10 November 1998, cols. 18-19, 40-45, 47-49, 75-77, and 87-89.	1-6, 9
X	CHONG, S. et al. Single-column purification of free recombinant proteins using a self-cleavable affinity tag derived from a protein splicing element. Gene. 19 June 1997, Vol. 192, No. 2, pages 271-281, especially Figure 1B at page 273 and pages 274-277.	9
--		----
Y	EVANS, T.C. et al. Semisynthesis of cytotoxic proteins using a modified protein splicing element. Protein Science. 05 November 1998, Vol. 7, No. 11, pages 2256-2264, especially Figure 1B at page 2258 and pages 2257 and 2259-2261.	1-6, 9
X,P		----
---		10
Y,P		

Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents	*T*	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y*	document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
D document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*&*	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

10 MARCH 2000

Date of mailing of the international search report

10 APR 2000

Name and mailing address of the ISA/US
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INTERNATIONAL SEARCH REPORT

International application No. PCT/US00/22776

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	MUIR, T.W. et al. Expressed protein ligation: A general method for protein engineering. Proceedings of the National Academy of Sciences, U.S.A. June 1998, Vol. 95, pages 6705-6710, see entire publication.	1-6, 9 ---- 7, 8, 10
X,P	EVANS, T.C. et al. The Cyclization and Polymerization of Bacterially Expressed Proteins Using Modified Self-splicing Inteins. The Journal of Biological Chemistry. June 1999, Vol 274, No. 26, pages 18359-18363, especially pages 18360-18362.	1-7, 9
Y	TELENTI, A. et al. The Mycobacterium xenopi GyrA Protein Splicing Element: Characterization of a Minimal Intein. Journal of Bacteriology. 14 October 1997, Vol. 179, No. 20, pages 6378-6382, especially pages 6379-6381.	8

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

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NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
 United States Patent and Trademark
 Office
 Box PCT
 Washington, D.C.20231
 ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 12 May 2000 (12.05.00)	
International application No. PCT/US99/22776	Applicant's or agent's file reference
International filing date (day/month/year) 30 September 1999 (30.09.99)	Priority date (day/month/year) 30 September 1998 (30.09.98)
Applicant XU, Ming-Qun et al	

1. The designated Office is hereby notified of its election made:

in the demand filed with the International Preliminary Examining Authority on:

02 February 2000 (02.02.00)

in a notice effecting later election filed with the International Bureau on:

2. The election was

was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer Maria Kirchner
Facsimile No.: (41 22) 740 14.35	Telephone No.: (41 22) 338 83.38

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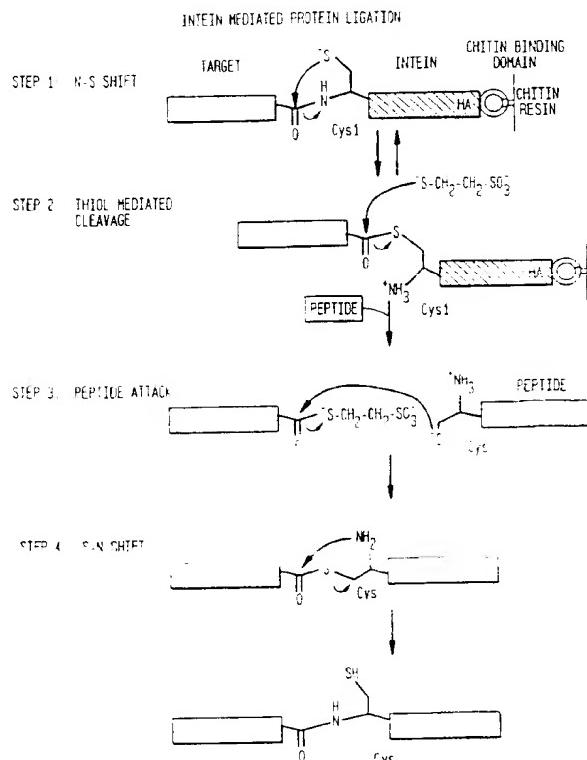
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : C12N		(11) International Publication Number: WO 00/1881
		A2
		(43) International Publication Date: 6 April 2000 (06.04.00)
<p>(21) International Application Number: PCT/US99/22776</p> <p>(22) International Filing Date: 30 September 1999 (30.09.99)</p> <p>(30) Priority Data: 60/102,413 30 September 1998 (30.09.98) US</p> <p>(71) Applicant (for all designated States except US): NEW ENGLAND BIOLABS, INC. [US/US]; 32 Tozer Road, Beverly, MA 01915 (US).</p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (for US only): XU, Ming-Qun [US/CN]; 40 Crescent Road, Hamilton, MA 01982 (US). EVANS, Thomas, C. [US/US]; 1st floor, 68 Albion Street, Somerville, MA 02143 (US).</p> <p>(74) Agent: WILLIAMS, Gregory, D.; New England Biolabs, Inc., 32 Tozer Road, Beverly, MA 01915 (US).</p>		
<p>(81) Designated States: CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</p> <p>Published <i>Without international search report and to be republished upon receipt of that report.</i></p>		
<p>(54) Title: INTEIN MEDIATED PEPTIDE LIGATION</p> <p>(57) Abstract</p> <p>In accordance with the present invention, there is provided a method for producing a semi-synthetic fusion protein <i>in vitro</i>, comprising the steps of producing a target protein fused to a protein splicing element (an intein) and selectively cleaving the fusion and ligating a synthetic protein or peptide at the C-terminal thioester of the target protein, which overcome many of the disadvantages and problems noted above. Specifically, the present invention has higher yields due to better thiol-induced cleavage with thiol-reagents which have been optimized for the ligation reaction, off-column ligation allows sample concentration and allows the use of less peptide. MESNA is an odorless thiol-reagent for ligation, and Mxe intein is from a bacterial source and often expresses better in bacterial cells. Furthermore, the present invention allows peptides to be directly ligated to the thioester bond formed between an intein and the target protein. The present invention also provides a method for producing a cytotoxic protein, comprising the steps of producing a truncated, inactive form of the protein <i>in vivo</i> which is fused to a protein splicing element, and selectively cleaving the fusion and ligating a synthetic protein or peptide at a C-terminal thioester of the target protein to restore the activity of the native cytotoxic protein. Recombinant vectors for producing such cleavable fusion proteins are also provided.</p>		



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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : C12N		A2	(11) International Publication Number: WO 00/18881
			(43) International Publication Date: 6 April 2000 (06.04.00)
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(22) International Filing Date: 30 September 1999 (30.09.99)			
(30) Priority Data: 60/102,413 30 September 1998 (30.09.98) US		Published <i>Without international search report and to be republished upon receipt of that report.</i>	
(71) Applicant (for all designated States except US): NEW ENGLAND BIOLABS, INC. [US/US]; 32 Tozer Road, Beverly, MA 01915 (US).			
(72) Inventors; and			
(75) Inventors/Applicants (for US only): XU, Ming-Qun [US/CN]; 40 Crescent Road, Hamilton, MA 01982 (US). EVANS, Thomas, C. [US/US]; 1st floor, 68 Albion Street, Somerville, MA 02143 (US).			
(74) Agent: WILLIAMS, Gregory, D.; New England Biolabs, Inc.. 32 Tozer Road, Beverly, MA 01915 (US).			

(54) Title: INTEIN MEDIATED PEPTIDE LIGATION

(57) Abstract

In accordance with the present invention, there is provided a method for producing a semi-synthetic fusion protein *in vitro*, comprising the steps of producing a target protein fused to a protein splicing element (an intein) and selectively cleaving the fusion and ligating a synthetic protein or peptide at the C-terminal thioester of the target protein, which overcome many of the disadvantages and problems noted above. Specifically, the present invention has higher yields due to better thiol-induced cleavage with thiol-reagents which have been optimized for the ligation reaction, off-column ligation allows sample concentration and allows the use of less peptide, MESNA is an odorless thiol-reagent for ligation, and Mxe intein is from a bacterial source and often expresses better in bacterial cells. Furthermore, the present invention allows peptides to be directly ligated to the thioester bond formed between an intein and the target protein. The present invention also provides a method for producing a cytotoxic protein, comprising the steps of producing a truncated, inactive form of the protein *in vivo* which is fused to a protein splicing element, and selectively cleaving the fusion and ligating a synthetic protein or peptide at a C-terminal thioester of the target protein to restore the activity of the native cytotoxic protein. Recombinant vectors for producing such cleavable fusion proteins are also provided.

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INTEIN MEDIATED PEPTIDE LIGATIONBACKGROUND OF THE INVENTION

5 Genetic engineering is a powerful approach to the manipulation of proteins. However, genetic methodologies are constrained by the use of only naturally coded amino acids. Furthermore, cytotoxic proteins are difficult to obtain by expression and isolation from a living source, since the
10 expression of the toxic protein can result in death of the host.

To some extent, protocols have been developed to circumvent these problems, for example, total chemical synthesis (Kent, S. B. (1988) *Ann. Rev. Biochem.* 57:957-989),
15 use of misacylated tRNAs (Noren, et al., (1989) *Science* 244:182-188), and semi-synthetic techniques (reviewed in Offord, R. (1987) *Protein Eng.* 1:151-157; Roy, et al. (1994)
Methods in Enzymol. 231:194-215; Wallace, C. J. (1993) *FASEB* 7:505-515). However, all of these procedures are limited by
20 either the size of the fragment which can be generated or by low reaction yield.

It would therefore be desirable to develop a high-yield,
semi-synthetic technique to allow *in vitro* fusion of a
25 synthetic protein or peptide fragment to an expressed protein without limitation as to the size of the fused fragments.

- 2 -

Likewise, in order to produce cytotoxic proteins, it would be desirable to develop a method of fusing a synthetic fragment, *in vitro*, to an inactive, expressed protein, so as to restore protein activity post-production from the host.

10

The modified Sce VMA intein has been used to generate thioester-tagged proteins for use in ligation (Example 19, U.S.S.N. 08/811,492, filed June 16, 1997; Chong, (1996) *J. Biol. Chem.*, 271(36):22159-22168; Chong, (1997) *Gene*, 192:271-281; and Muir, et al. (1998) *Proc. Natl. Acad. Sci USA* 95:6705-6710).

15

Some disadvantages have been low yields due to poor cleavage of the Sce VMA intein with thiol-reagents that are optimum for ligation, the need for large peptide quantities due to on-column reactions, the use of odoriferous reagents, and/or low protein yields due to the use of a large, eukaryotic intein.

20

SUMMARY OF THE INVENTION

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In accordance with the present invention, there is provided a method for producing a semi-synthetic fusion protein *in vitro*, comprising the steps of producing a target protein fused to a protein splicing element (an intein) and selectively cleaving the fusion and ligating a synthetic

protein or peptide at the C-terminal thioester of the target protein, which overcome many of the disadvantages and problems noted above. Specifically, the present invention has higher yields due to better thiol-induced cleavage with thiol reagents which have been optimized for the ligation reaction.

5 Off-column ligation allows for sample concentration as well as the use of less peptide. In a particularly preferred embodiment, thiol reagents such as 2-mercaptopethanesulfonic acid (MESNA), which is an odorless thiol-reagent, is used for cleavage and ligation along with the Mxe intein, which is from a bacterial source and often expresses better in bacterial cells. Furthermore, the present invention allows peptides to be directly ligated to the thioester bond formed between an intein and the target protein. The present invention also

10 provides a method for producing a cytotoxic protein, comprising the steps of producing a truncated, inactive form of the protein *in vivo* which is fused to a protein splicing element, and selectively cleaving the fusion and ligating a synthetic protein or peptide at a C-terminal thioester of the target protein to restore the activity of the native cytotoxic protein.

15 Recombinant vectors for producing such cleavable fusion proteins are also provided.

20

BRIEF DESCRIPTION OF THE DRAWINGS

25

Figure 1 is a flow diagram depicting the chemical reactions which enable intein-mediated peptide ligation. The

thioester generated at the C-terminus of the target protein during IMPACT™ purification was used in a 'native chemical ligation' reaction. This allowed the ligation of a synthetic peptide to a bacterially expressed protein. A typical ligation reaction involved the expression of the target protein-intein-CBD fusion followed by binding to a chitin resin. A thiol reagent induced cleavage of the intein. The target was eluted from the chitin resin and a synthetic peptide was added. The ligation reaction proceeded overnight.

10

Figure 2 is a gel depicting the results of cleavage and ligation reactions using various thiols. Cleavage and ligation reactions with different thiols visualized on 10-20% Tricine gels. MYB (a fusion protein of maltose binding protein-Sce VMA intein (N454A)-chitin binding domain) and MXB (a fusion protein of maltose binding protein-Mxe GyrA (N198A) intein-chitin binding domain) were incubated overnight at 4°C with various thiols (50 mM) in 150 mM Tris, 100 mM NaCl, pH 8 in the presence of a 30 amino acid peptide with an N-terminal cysteine. The peptide ligates to the C-terminus of MBP. Lanes 1-5 ligation with MYB. Lane 1 no thiol. Lane 2 dithiothreitol. Lane 3 2-mercaptopethanesulfonic acid. Lane 4 3-mercaptopropionic acid. Lane 5 thiophenol. Lanes 6-10 ligation with MXB. Lane 6 no thiol. Lane 7 dithiothreitol. Lane 8 2-mercaptopethanesulfonic acid. Lane 9 3-mercaptopropionic acid. Lane 10 thiophenol.

25

Figure 3 is a gel depicting direct ligation of a peptide to the thioester formed between the Sce VMA intein and maltose binding protein. SDS-PAGE of direct ligation reaction with a 10-20% Tricine gel. Lane 1: a precursor protein (MYBleu) consisting of maltose binding protein-Sce VMA1 intein-chitin binding domain was heated to >95°C for 5 minutes in a buffer of 50 mM Trizma base, pH 8.5 containing 100 mM NaCl, 1% SDS, and mM tris-(2-carboxyethyl)phosphine (TCEP) followed by overnight incubation at room temperature. The precursor (MYBleu) is visible along with the Sce VMA1 intein (Y) and maltose binding protein (M), which are cleavage products. Lane 2: the precursor protein was subjected to the same conditions as described in Lane 1 except that the 30 amino acid peptide (1 mM) was added. The precursor (MYB) and cleavage products (Y and M) are visible along with the ligation product (M+30mer) formed when the 30 amino acid peptide fuses to maltose binding protein.

Figure 4 is a diagram depicting the pTXB1 expression vector of Example I (SEQ ID NO:7 and SEQ ID NO:8).

Figure 5 is the DNA sequence of pTXB1 (SEQ ID NO:5).

Figure 6 is a gel depicting the results of the *Hpa*I protein ligation reaction. Protein ligation reactions examined on 10-20% Tricine gels. Lane 1: clarified cells extract after IPTG (0.5 mM) induction of ER2566 cells containing the pTXB2-*Hpa*I

plasmid. The fusion protein of *HpaI₂₂₃*-Mxe GyrA-intein-CBD (52 kDa) is visible. Lane 2: cell extract as in Lane 1 after passage over a chitin column, which results in the binding of the fusion protein. Lane 3: *HpaI₂₂₃* (25.7 kDa) after cleavage from the fusion protein by addition of MESNA. Lane 4: ligation product of *HpaI₂₂₃* (0.2 mg/mL) with 1 mM of a 31 amino acid peptide (ligation product 29.6 kDa), representing the residues necessary to generate full length *HpaI*, after overnight incubation at 4°C. Lane 5: full length *HpaI* from a recombinant source (29.6 kDa) containing BSA (66 kDa) and two impurities.

Figure 7 is a western blot of various proteins ligated to a biotinylated peptide. Proteins purified with the Mxe GyrA IMPACT™ derivative were ligated to a synthetic peptide which contained an antibody recognition sequence.

DETAILED DESCRIPTION OF THE INVENTION

The ligation methods of the present invention are based on the discovery that a cysteine or peptide fragment containing an N-terminal cysteine may be fused, *in vitro*, to a bacterially expressed protein produced by thiol-induced cleavage of an intein (U.S. Patent No. 5,496,714, Example 19 of U.S.S.N. 08/811,492 filed June 16, 1997, Chong, et al., (1996) *supra* and Chong, et al., (1997) *supra*.

The ligation procedure disclosed herein utilizes a protein splicing element, an intein (Perler, et al., (1994) *Nucleic Acids Res.* 22:1125-1127) to precisely create a thioester at the C-terminal α -carbon of an expressed protein. ~

5 This reactive thioester could be present between the target protein and intein or generated by the addition of a thiol reagent. Previously the generation such a thioester was described using an intein (CIVPS) that was modified to undergo thiol inducible cleavage at its N-terminal junction in

10 the presence of thiol reagent dithiothreitol (DTT) (Chong, et al. (1997) *supra*; Comb, et.al. U.S. Patent No. 5,496,714). This C-terminal thioester was previously used in a 'native' chemical ligation' type reaction (Dawson, et al., (1994) *Science* 266:776-779) to fuse ^{35}S -cysteine or a peptide

15 fragment containing an N-terminal cysteine to a bacterially expressed protein (Example 19, Comb, et.al. U.S. Patent No. 5,834,247, Chong (1996) *supra* and Chong (1997) *supra*.

The ligation method of the instant invention begins with

20 the purification of the thioester-tagged target protein using an intein as described (Chong, et.al. (1997) *supra*). The direct ligation method of the instant invention begins with the isolation of a precursor composed of the target protein-
intein-CBD. In one preferred embodiment, the host cell is
25 bacterial. In other embodiments the host cell may be yeast,
insect, or mammalian. A cysteine thiol at the N-terminus of a synthetic peptide nucleophilicly attacks a thioester present

on the freshly isolated C-terminal α -carbon of the target protein or directly attacks the thioester present between the target protein and intein. This initially generates a thioester between the two reactants which spontaneously rearranges
5 into a native peptide bond (Figure 1).

In order to optimize the ligation efficiency so that greater than 90% of the bacterially expressed target protein can be fused to the synthetic peptide or protein, specific thiol reagents and inteins are screened. In a preferred embodiment, the intein may be any CIVPS, such as *Sce* VMA,
10 *Mxe* GyrA or derivatives of mutants thereof, and the thiol reagent is 2-mercaptop-ethanesulfonic acid, thiophenol, DTT, or 3-mercaptopropionic acid (Comb, et al., U.S. Patent No.
15 5,496,714; U.S. Patent No. 5,834,247).

In one particularly preferred embodiment, an intein whose protein splicing activity has been blocked by mutation is utilized. The mutant must, however, retain the ability to undergo the N-S shift, thus allowing thioester formation between itself and an N-terminal protein. This thioester can then be nucleophilically attacked by a thiol reagent or by the N-terminal cysteine of a peptide sequence. For example, by mutating the C-terminal asparagine (asn 198) of an intein
20 from the GyrA gene of *Mycobacterium xenopi* (Telenti, et al.,
25 (1997) *J Bacteriol* 179:6378-6382) to an alanine created a

thiol inducible cleavage element. This modified intein cleaved well with thiol reagents that were optimal for the ligation reaction, such as MESNA and thiophenol. Furthermore, optimal thiol reagent and intein combinations can be determined by 5 incubating a precursor protein containing the intein of interest with a wide variety of thiol reagents followed by determination of the extent of cleavage of the precursor protein (Figure 2).

10 The use of such intein and specific thiol reagents leads to optimal yields and high ligation efficiencies; typically greater than 90% of the N-terminal ligation fragment can be modified.

15 The ligation methods of the present invention expand the ability to incorporate non-coded amino acids into large protein sequences by generating a synthetic peptide fragment with fluorescent probes, spin labels, affinity tags, radiolabels, or antigenic determinants and ligating this to an 20 *in vivo* expressed protein isolated using a modified intein.

Furthermore, this procedure allows the isolation of cytotoxic proteins by purifying an inactive truncated precursor from a host source, for example bacteria, and 25 generating an active protein or enzyme after the ligation of a synthetic peptide. For example, restriction endonucleases which have not successfully been cloned by traditional

methods may be produced in accordance with the present invention.

Also, the direct ligation procedure allows the ligation of -
5 a protein or peptide sequence to another protein or peptide sequence without the use of exogenous thiol reagents. Direct ligation relies on the nucleophilic attack of the N-terminal amino acid of one peptide on the thioester formed between a target protein and an intein (Figure 3).

10

In summary, a fusion protein can be created using the methods of the present invention that possesses unique properties which, currently, can not be generated genetically.

15

The Examples presented below are only intended as specific preferred embodiments of the present invention and are not intended to limit the scope of the invention. The present invention encompasses modifications and variations of the methods taught herein which would be obvious to one of ordinary skill in the art.

20

The references cited above and below are herein incorporated by reference.

EXAMPLE I**Creation of vectors pTXB1 and pTXB2 for ligation:**

5 Asparagine 198 of the *Mxe* GyrA intein (Telenti, et al.,
(1997) *J Bacteriol.* 179:6378-6382) was mutated to alanine by
linker insertion into the *XmnI* and *PstI* sites of
pmxeMIPTyrXmnSPdel to create pMXP1. The *XmnI* site was
originally introduced into the unmodified *Mxe* GyrA intein
10 sequence by silent mutagenesis. The *PstI* site was a unique
site in the plasmid. The linker was composed of mxe#3 (5'-
GGTCGTCAGCCACGCTACTGGCCTACCGGTTGATAGCTGCA-3')
(SEQ ID NO:1) and mxe#4 (5'-GCTATCAACCGGTGAGGCCAGTAG
CGTGGCTGACGAACC-3') (SEQ ID NO:2).

15 Into pMXP1 another linker composed of mxe#1 (5'-TC
GAATCTAGACATATGGCCATGGGTGGCGGCCGCTCGAGGGCTCTCC
TGCATCACGGGAGATGCA-3') (SEQ ID NO:3) and mxe#2 (5'-CTAG
TGCATCTCCCGTGATGCAGGAAGAGCCCTCGAGGCGHGCGCCACCCA
20 TGGCCATATGTCTAGAT-3') (SEQ ID NO:4) was inserted into the
XbaI and *SpeI* sites to introduce a multiple cloning site (*XbaI*-
NdeI-*NcoI*-*NotI*-*XbaI*-*SapI*) before the *Mxe* GyrA intein (pMXP2).

25 The 0.6 kilobase *NotI* to *AgeI* fragment of pMXP2 was
ligated into the same sites in pTYB1 (IMPACT kit, New England
Biolabs, Beverly, MA) and the *NcoI* to *AgeI* fragment of pMXP2

was cloned into pTYB3 (IMPACT kit, New England Biolabs, Beverly, MA) to create plasmids pTXB1 (see Figure 4 and 5) (SEQ ID NO:5) and pTXB2, respectively. These vectors have a multiple cloning site upstream of the modified *Mxe* GyrA intein-chitin binding domain fusion. This allows the insertion of a target gene of interest inframe with the intein and chitin binding domain (CBD).

Creation of vectors pMYBleu for ligation:

pMYBleu was as described in Chong, et al., (1998), *J. Biol. Chem.* 273:10567-10577. This vector consisted of maltose binding protein upstream of the Sce VMA intein-chitin binding domain. A leucine is present at the -1 position instead of the native residue (which is a glycine).

Purification of Thioester-Tagged Proteins:

Protein purification was as described using the Sce VMA intein (Chong, et.al., (1997) *Gene* 192:271-281) with slight modification. ER2566 cells (IMPACT T7 instruction manual from New England Biolabs, Beverly, MA) containing the pTXB vector with the appropriate insert were grown to an OD₆₀₀ of 0.5-0.6 at 37°C at which point they were induced with 0.5 mM IPTG overnight at 15°C. Cells were harvested by centrifugation and lysed by sonication (performed on ice). The

three part fusion protein was bound to chitin beads (10 mL bed volume, Figure 6, lanes 1 and 2) equilibrated in Buffer A (50 mM Tris, pH 7.4, and 500 mM NaCl), and washed with 10 column volumes of Buffer A to remove unbound material.

5

Cleavage was initiated using a buffer of 50 mM 2-mercaptoethanesulfonic acid (MESNA), 50 mM Tris, pH 8.0 and 100 mM NaCl. Other thiol reagents were also used at other times, such as thiophenol, dithiothreitol, and/or 3-mercaptopropionic acid. After overnight incubation at from 4-25°C protein was eluted from the column (Figure 6 lane 3). This protein contained a thioester at the C-terminus.

10

Purification of MYB, MYBleu and MXB:

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20

25

Full length precursor proteins consisting of maltose binding protein-Sce VMA intein (N454A)-chitin binding domain (MYB) and maltose binding protein-Mxe GyrA (N198A) intein-chitin binding domain (MXB) were purified after induction and sonication, as described above, by applying the sonicated sample to a 10 mL column of amylose resin (New England Biolabs, Beverly, MA). Unbound proteins were washed from the column with 10 column volumes of Buffer A (see purification of thioester-tagged proteins). Bound proteins were eluted with a buffer of 50 mM Tris, pH 8, containing 100 mM NaCl and 10 mM maltose. Fractions were collected and protein

concentrations were determined using the Bio-Rad Protein Assay (Hercules, CA).

Peptide Synthesis:

5

Peptides for subsequent ligation reactions were synthesized on an ABI model 433A peptide synthesizer utilizing *FastMoc*TM chemistry (Fields, et al., (1991) *Pept Res* 4, 95-101) at a 0.085 mmol scale. Preloaded HMP (p-hydroxymethylphenoxyethyl) polystyrene resins (Applied Biosystems, Foster City, CA) functionalized at 0.5 mmol/g was used in conjunction with Fmoc/NMP chemistry utilizing HBTU amino acid activation (Dourtoglou, et al., (1984) *Synthesis* 572-574; Knorr, et al., (1989) *Tetrahedron Lett* 30, 1927-1930). Fmoc amino acids were purchased from Applied Biosystems (Foster City, CA).

Synthesis proceeded with a single coupling during each cycle. Peptide cleavage from the resin and simultaneous removal of side chain protecting groups was facilitated by the addition of cleavage mixture (Perkin Elmer, Norwalk, CT) consisting of 0.75 g phenol, 0.25 mL 1,2-ethanedithiol, 0.5 mL deionized H₂O, and 10 mL TFA. The resin was flushed with nitrogen and gently stirred at room temperature for 3 hours. Following filtration and precipitation into cold (0°C) methyl-t-butyl ether, the precipitate in the ether fraction was

collected by centrifugation. The peptide precipitate was vacuum dried and analyzed by mass spectrometry using a Perceptive Biosystems (Framingham, MA) MALDI-TOF mass spectrometer.

5

Final purification was by HPLC using a Waters HPLC system with a Lambda-Max Model 481 Multiwavelength detector (set at 214 nm), 500 series pumps and automated gradient controller with a Vydac semi-preparative C18 column. Elution of the peptide was with a 60 minute linear gradient of 6-60% acetonitrile (v/v) in an aqueous solution of 10 0.1% TFA (v/v).

Protein Cleavage and Ligation Reactions:

15

Cleavage of MYB and MXB: The precursor protein (1 mg/mL) was incubated overnight at 4°C with or without a thiol reagent (50 mM) in 150 mM Tris, pH 8, containing 100 mM NaCl.

20

Ligation reactions with MYB and MXB: The precursor protein (1 mg/mL) was treated as described for cleavage except that a 30 amino acid peptide (1 mM final concentration, NH₂-CAYKTTQANKHIIIVACEGNPYVPVHFDASV-COOH (SEQ ID NO:6) was also included in the reaction (Figure 2).

25

Ligation reactions after purification of thioester-tagged proteins: Lyophilized peptides (New England Biolabs, Beverly, MA) were added (to 1 mM final concentration) directly to the thioester-tagged protein freshly isolated from the chitin column. The reaction was allowed to proceed overnight at 5 from 4-25°C. In both ligation procedures the condensation of the reactants is visible on a 10-20% Tricine gel (Figure 6). The ligation reaction was tested in conditions of 5-150 mM Tris or HEPES buffers, 50-1000 mM NaCl, 10 mM Maltose, and 10 pH 6-11 and 0-6 M Urea.

Direct Ligation Reactions:

MYBleu (1 mg/mL) was incubated in 6 M Urea or 1% SDS, 15 pH 7.5-8.5, 50-200 mM NaCl, and 1 mM of a 30 amino acid peptide ($\text{NH}_2\text{CAYKTTQANKHIVVACEGNPYVPVHFDASV-COOH}$ (SEQ ID NO:6)). The MYBleu was incubated for 0-180 minutes at either 4°C or 100°C prior to the addition of the 30 amino acid peptide. Ligation reactions proceeded overnight at either 4°C 20 or 25°C.

EXAMPLE II**Labeling a target protein: Maltose Binding Protein**

5 Maltose binding protein (MBP, 42 kDa) was isolated as described in Example I above using the IMPACT procedure (IMPACT manual from New England Biolabs, Inc., Beverly, MA) in the presence of MESNA.

10 A biotinylated peptide possessing an N-terminal cysteine (CDPEK*DS-COOH (SEQ ID NO:9)), in which the biotin was attached to the ϵ -amino group of the lysine residue) was ligated to the freshly purified target protein as described above. Briefly, 4 μ L of biotinylated peptide (10 mM) were mixed with a 36 μ L aliquot of the freshly purified MBP sample. 15 The mixture was incubated at 4°C overnight.

20 Western blots with alkaline phosphatase linked anti-biotin antibody detected the presence of the ligated product but not the unligated target protein (Figure 7). The efficiency of the ligation is typically greater than 90% when MESNA is used for cleavage.

EXAMPLE III**Labeling a target protein: Bst DNA Polymerase I Large Fragment (Bst Pol 1)**

5

Bst DNA Polymerase I large fragment (67 kDa) was isolated as described in Example I above using the IMPACT procedure (IMPACT manual from New England Biolabs, Inc., Beverly, MA) in the presence of MESNA.

10

A biotinylated peptide possessing an N-terminal cysteine (CDPEK*DS-COOH (SEQ ID NO:9)), in which the biotin was attached to the ϵ -amino group of the lysine residue) was ligated to the freshly purified target protein as described.

15

Briefly, 4 μ L of biotinylated peptide (10 mM) were mixed with a 36 μ L aliquot of the freshly purified Bst Pol 1 sample. The mixture was incubated at 4°C overnight.

20

Western blots with alkaline phosphatase linked anti-biotin antibody detected the presence of the ligated product but not the unligated target protein (Figure 7). The efficiency of the ligation is typically greater than 90% when MESNA is used for cleavage.

EXAMPLE IV**Labeling a target protein: Paramyosin**

5 Paramyosin (29 kDa) was isolated as described in Example I above using the IMPACT procedure (IMPACT manual from New England Biolabs, Inc., Beverly, MA) in the presence of MESNA.

10 A biotinylated peptide possessing an N-terminal cysteine (CDPEK^{*}DS-COOH (SEQ ID NO:9)), in which the biotin was attached to the ϵ -amino group of the lysine residue) was ligated to the freshly purified target protein as described. Briefly, 4 μ L of biotinylated peptide (10 mM) were mixed with 15 a 36 μ L aliquot of the freshly purified paramyosin sample. The mixture was incubated at 4°C overnight.

20 Western blots with alkaline phosphatase linked anti-biotin antibody detected the presence of the ligated product but not the unligated target protein (Figure 7). The efficiency of the ligation is typically greater than 90% when MESNA is used for cleavage.

EXAMPLE V**Labeling a target protein: *E. coli* Thioredoxin**

5 *E. coli* thioredoxin (12 kDa) was isolated as described in Example I above using the IMPACT procedure (IMPACT manual from New England Biolabs, Inc., Beverly, MA) in the presence of MESNA.

10 A biotinylated peptide possessing an N-terminal cysteine (CDPEK^{*}DS-COOH (SEQ ID NO:9)), in which the biotin was attached to the ϵ -amino group of the lysine residue) was ligated to the freshly purified target protein as described. Briefly, 4 μ L of biotinylated peptide (10 mM) were mixed with 15 a 36 μ L aliquot of the freshly purified thioredoxin sample. The mixture was incubated at 4°C overnight.

20 Western blots with alkaline phosphatase linked anti-biotin antibody detected the presence of the ligated product but not the unligated target protein (Figure 7). The efficiency of the ligation is typically greater than 90% when MESNA is used for cleavage.

EXAMPLE VI**Isolation of a cytotoxic protein:**

5 The ligation procedure of Example I was applied to the isolation of a potentially cytotoxic protein. An endonuclease from *Haemophilus parainfluenzae* (*Hpal*; Ito, et al., (1992) *Nucleic Acids Res* 20:705-709) was generated by ligating an inactive truncated form of the enzyme expressed in *E. coli* (ER2566 cells, New England Biolabs, Inc., Beverly, MA) with 10 the missing amino acids that were synthesized chemically.

15 The first 223 amino acids of *Hpal* (full length *Hpal* is 254 amino acids) were fused in frame with the modified *Mxe* GyrA intein and the CBD. The 223 amino acid *Hpal* fragment was isolated as described for purification of thioester tagged proteins. The truncated *Hpal* displayed no detectable enzymatic activity.

20 A synthetic peptide representing the 31 amino acids needed to complete *Hpal* was ligated onto the 223 amino acid truncated form of *Hpal* by the method of Example I.

Enzymatic Assay for *HpaI*:

The activity of the fused *HpaI* was determined by its ability to digest Lambda DNA (New England Biolabs, Beverly, MA). Serial dilutions of ligated or truncated *HpaI*, with the appropriate peptide added to 1 mM, were incubated with 1 μ g of Lambda DNA for 1 hour at 37°C in a buffer of 20 mM Tris-acetate, pH 7.9, 10 mM magnesium acetate, 50 mM potassium acetate, 1 mM dithiothreitol, and 170 μ g/mL BSA (total volume 30 μ L). Digestion reactions were visualized on 1% agarose gels permeated with ethidium bromide. One unit of *HpaI* was defined as the amount of enzyme necessary to digest 1 μ g of Lambda DNA in one hour at 37°C.

The newly ligated *HpaI* had a specific activity of 0.5- 1.5×10^6 units/mg which correlated well with the expected value of $1-2 \times 10^6$ units/mg for the full length enzyme.

WHAT IS CLAIMED IS:

1. A method for fusing an expressed protein with a peptide, said method comprising the steps of:

5 (a) generating at least one C-terminal thioester-tagged target protein;

(b) generating at least one target peptide having a specified N-terminal; and

(c) ligating said target peptide to said target protein.

10

2. The method of claim 1, wherein said target protein is generated from a first plasmid comprising an intein having N-terminal cleavage activity.

15

3. The method of claim 2, wherein said intein comprises an intein having a cysteine residue at the N-terminal of the intein.

20

4. The method of claim 3, wherein said target protein is generated by thiol reagent-induced cleavage of said intein.

25

5. The method of claim 4, wherein said thiol reagent is selected from the group consisting of MESNA, thiophenol, DTT, β -mercaptoethanol or derivatives thereof.

6. A fusion protein produced by the method of any one of claims 1-5.

7. A cyclic protein produced by the method of claim 1.
8. A modified intein comprising a mutant Mxe GyrA intein capable of thiol reagent-induced cleavage to produce a thioester at the C-terminal of an adjacent target protein.
5
9. A method of generating a reactive thioester comprising contacting a thiol reagent selected from the group consisting essentially of MESNA, thiophenol, DTT, β -mercaptoethanol or derivatives thereof with a precursor comprising a target protein and intein.
10
10. A method for screening thiol reagents which cleave a target intein comprising the steps of:
 - (a) isolating a precursor comprising a protein and a modified intein;
 - (b) contacting a thiol reagent with the precursor of step (a);
20
 - (c) determining whether a splicing or cleaving event occurs.
11. The method of claim 10, comprising the further step of determining whether the spliced or cleaved product of step (c) can ligate to a target peptide having an N-terminal cytokine.
25

Figure 1
Intein Mediated Protein Ligation

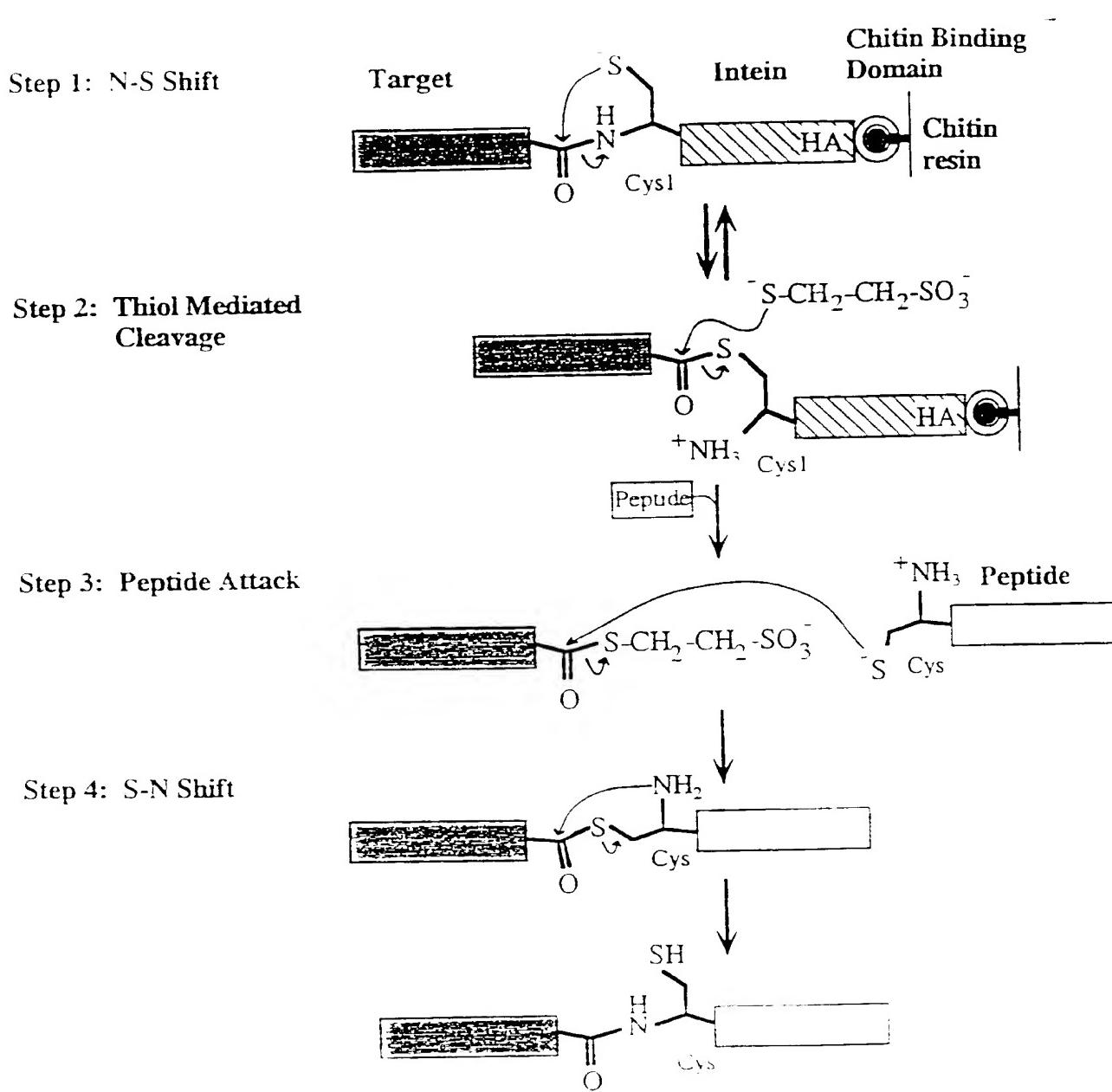


Figure 2

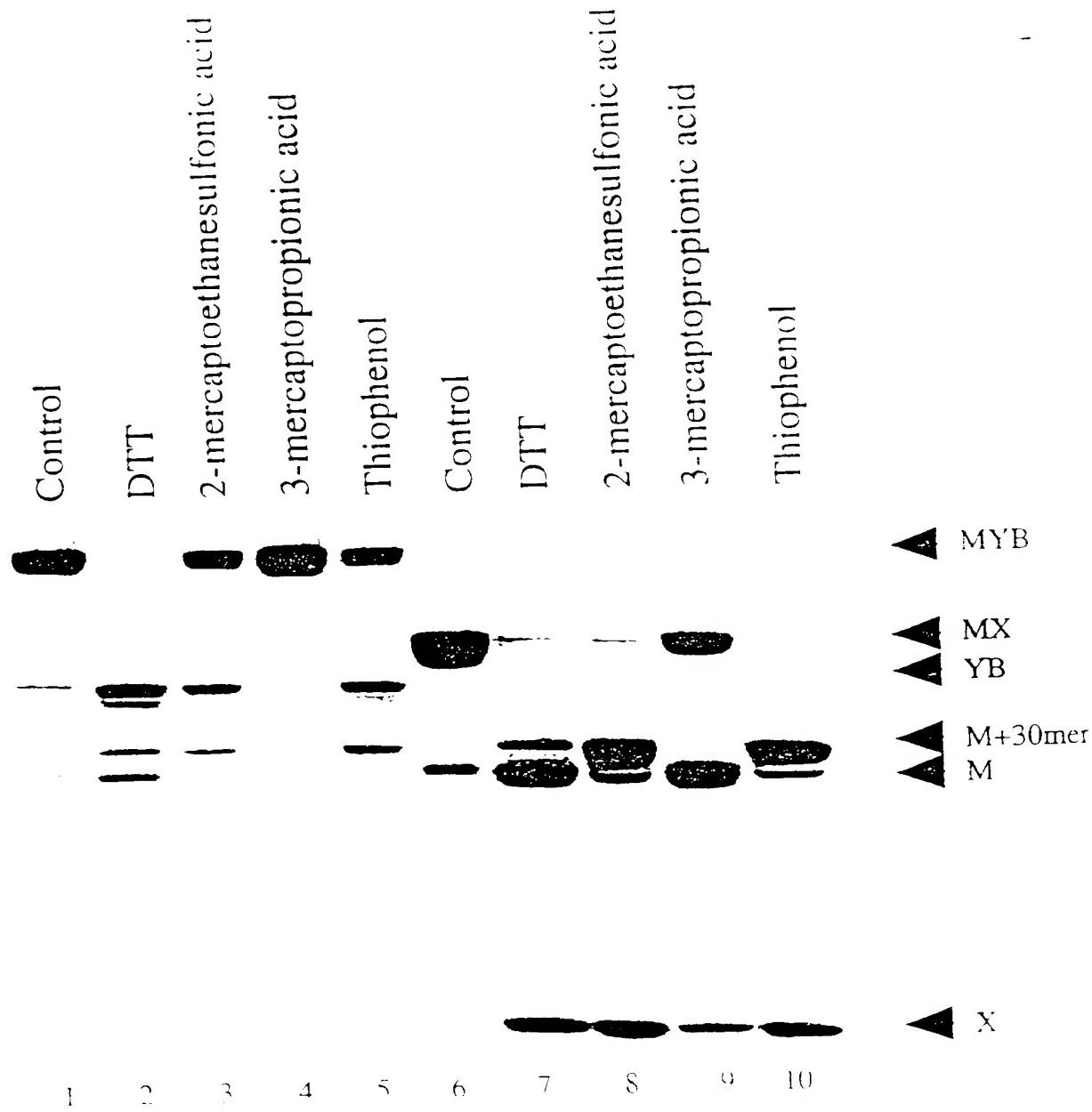
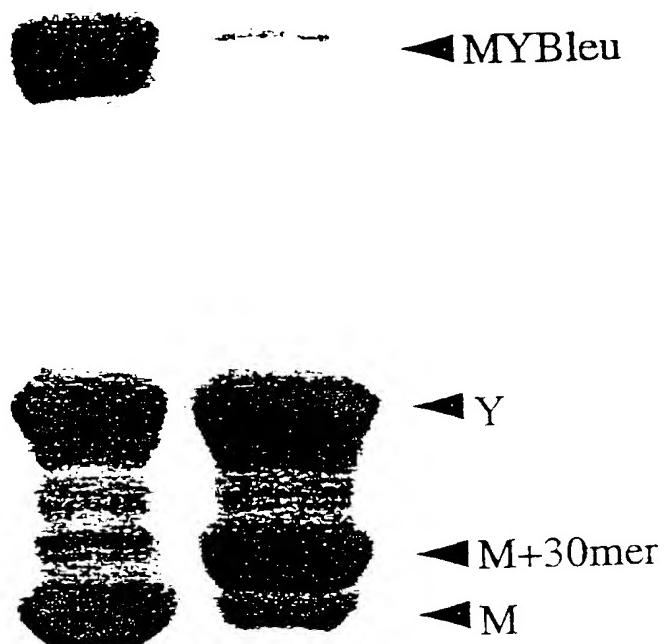
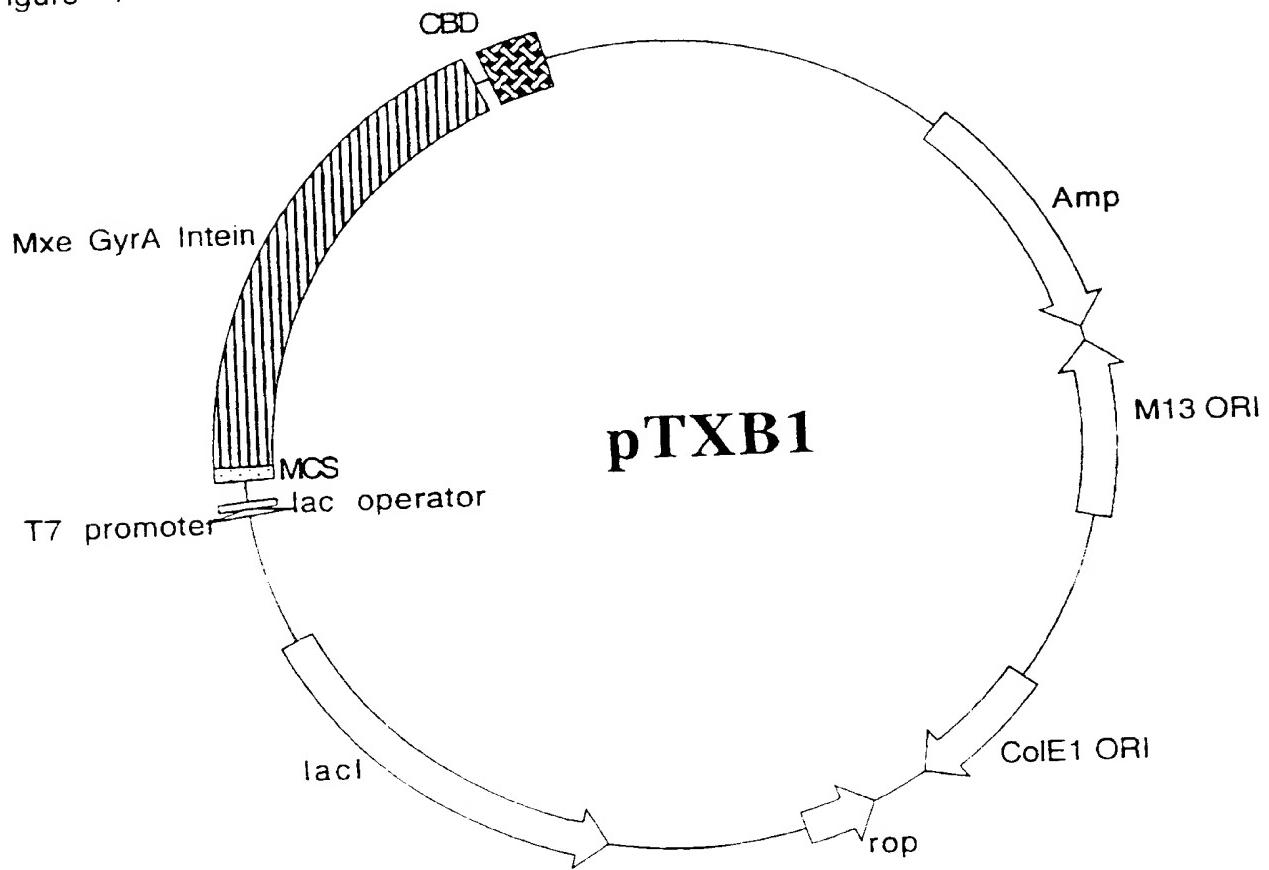


Figure 3. Direct Ligation Reaction



pTXB1 Expression Vector

Figure 4



pTXB1
....Met Ala Met Gly Gly Gly Ara Leu Glu Gly Ser Ser Cysl...Intein
....Met Ala Met Gly Gly Gly Ara Leu Glu Gly Ser Ser Cysl...Intein
CAT ATG GGC ATG GGT GGC GGC CGC CTC GAG GGC TCT TCC TGC
NdeI NcoI NotI XbaI SapI

Figure 5

1 AACTACGTCA GGTGGCACTT TTTCGGGAAA TGTGGCGGGAA ACCCCTATTT
51 GTTTATTTTT CTAATACAT TCAAATATGT ATCCGCTCAT GAGACAATAA
101 CCCTGATAAA TGCTTCATA ATAATTGAAAA AGGAAGAGTA TGAGTATTCA
151 ACATTTCCGT GTCGCCCTTA TTCCCTTTTG TGCGGCATTT TGCCTTCCTG
201 TTTTTGCTCA CCCAGAAACG CTGGTGAAG TAAAAGATGC TGAAGATCAG
251 TTGGGTGCAC GAGTGGGTAA CATCGAACTG GATCTCAACA GCGGTAAGAT
301 CCTTGAGAGT TTTCGCCCCG AAGAACGTTT TCCAATGATG AGCACTTTTA
351 AAGTTCTGCT ATGTGGCGCG GTATTATCCC GTGTTGACGC CGGGCAAGAG
401 CAACTCGGTC GCCGCATACA CTATTCTCAG AATGACTTGG TTGAGTACTC
451 ACCAGTCACA GAAAAGCATC TTACGGATGG CATGACAGTA AGAGAATTAT
501 GCAGTGCCTGC CATAACCATG AGTGATAACA CTGCGGCCAA CTTACTTCTG
551 ACAACGGATCC GAGGACCGAA CGAGCTAACG CCTTTTTTGC ACAACATGGG
601 GGATCATGTA ACTCGGCTTC ATCGTTGGGA ACCGGAGCTG AATGAAGCCA
651 TACCAAACGA CGAGCGTGAC ACCACGATGC CTGTAGCAAT GGCAACAAACG
701 TTGCGCAAAC TATTAACTGG CGAACTACTT ACTCTAGCTT CCCGGCAACA
751 ATTAATAGAC TGGATGGAGG CGGATAAAAGT TGCGAGGACCA CTTCTGCGCT
801 CGGCCCTTCC GGCTGGCTGG TTTATTGCTG ATAAATCTGG AGCCGGTGAG
851 CGTGGGTCTC CGGGTATCAT TGCAGCACTG GGGCCAGATG GTAAGCCCTC

901 CCGTATCGTA GTTATCTACA CGACGGGAG TCAGGCACT ATGGATGAAC
951 GAAATAGACA GATCGCTGAG ATAGGTGCCT CACTGATTAA GCATTGGTAA
1001 CTGTCAGACC AAGTTTACTC ATATATACTT TAGATTGATT TACCCCGGTT
1051 GATAATCAGA AAAGCCCCAA AAACAGGAAG ATTGTATAAG CAAATATTTA
1101 AATTGTAAAC GTTAATATTT TGTTAAAATT CGCGTTAAAT TTTTGTAAA
1151 TCAGCTCATT TTTAACCAA TAGGCCAAA TCGGCAAAT CCCTTATAAAA
1201 TCAAAAGAAT AGCCCGAGAT AGGGTTGAGT GTTGTCCAG TTTGGAACAA
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1401 CCGATTAGA GCTTGACGGG GAAAGCCGGC GAACGTGGCG AGAAAGGAAG
1451 GGAAGAAAGC GAAAGGAGCG GGCCTAGGG CGCTGGCAAG TGTAGCGGTC
1501 ACCCTGCGCG TAACCACCAAC ACCCGCCGCG CTTAATGCGC CGCTACAGGG
1551 CGCGTAAAG GATCTAGCTG AAGATCTTT TTGATAATCT CATCACCAAA
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1651 GATCAAAGGA TCTTCTTGAG ATCCTTTTT TCTGCGCGTA ATCTGCTGCT
1701 TGCAAACAAA AAAACCACCG CTACCAGCGG TGGTTGTTT GCCGGATCAA
1751 GAGCTACCAA CTCTTTTCC GAAGGTAACG GGCTTCAGCA GAGCGCAGAT
1801 ACCAAATACT GTCTTCTAG TGTAGCCGT A GTTAGGCCAC CACTTCAAGA
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1951 ATAGTTACCG GATAAGGCCG AGCGGTCGGC CTGAACGGGG GGTTCGTGCA
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2251 GAAAAACGCC AGCAACGCAG CCTTTTACG GTTCCTGGCC TTTTGTGGC
2301 CTTTTGCTCA CATGTTCTTT CCTGGTTAT CCCCTGATTC TGTGGATAA'

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2351 CGTATTACCG CCTTTGACTG AGCTGATACC GCTGCCGCA GCCGAACGAC
2401 CGAGCCGAGC GAGTCAGTGA GCGAGGAAGC TATGGTGCAC TCTCAGTACA
2451 ATCTGCTCTG ATGCCGCATA GTTAAGCCAG TATAACACTCC GCTATCGCTA
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2551 GCCCTGACGG GCTTGTCTGC TCCCGGCATC CGCTTACAGA CAAGCTGTGA
2601 CCGTCTCCGG GAGCTGCATG TGTCAGAGGT TTTCACCGTC ATCACCGAAA
2651 CGCGCGAGGC AGCTGCGGTA AAGCTCATCA GCGTGGTCGT GCAGCGATTG
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2751 GCGTTAATGT CTGGCTCTG ATAAAGCGGG CCATGTTAAG GGCGGTTTTT
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2851 GGGGTAATGA TACCGATGAA ACGAGAGAGG ATGCTCACGA TACGGGTTAC
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2951 CGGTATGGAT GCGGCGGGAC CAGAGAAAAA TCACTCAGGG TCAATGCCAG
3001 ccgaACGCCA GCAAGACGTA GCCCAGCGCC TCGGCCGCCA TGCCGGCGAT
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3101 CTTGAGCGAG GGCGTGAAG ATTCCGAATA CCGCAAGCGA CAGGCCGATC
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3251 CGGCGACGAT AGTCATGCC CGCGCCCACC GGAAGGAGCT GACTGGGTTG
3301 AAGGCTCTCA AGGGCATCGG TCGAGATCCC GGTGCCTAAT GAGTGAGCTA
3351 ACTTACATTA ATTGGTGTGC GCTCACTGCC CGCTTCCAG TCGGGAAACC
3401 TGTCGTGCCA GCTGCATTAA TGAATCGGCC AACGCGCGG GAGAGGGCGT
3451 TTGCGTATTG GGCGCCAGGG TGGTTTTCT TTTCACCAGT GAGACGGGCA
3501 ACAGCTGATT GCCCTTGACG CGCTGGCCT GAGAGACTTG CAGCAAGCGG
3551 TCCAAUGCTGA TTTGCCCGAG CAGCCGAAAGA TCCCTGTTGA TGGTGGTTAA
3601 CGGCGGGATA TAACATGAGC TGTCTTGGGT ATCGTCGTAT CCCACTACCG
3651 AGATATCCGC ACCAACCGCGC AGCCCCGACT CGGTAATGCC GCGCATTCGCG
3701 CCCAGCGGCCA TCTGATCGTT GGCAACCAGC ATCGCAGTGG GAACGATGCC
3751 CTCATTTCAGC ATTTGCATGG TTTCTTGAAA ACCGGACATG GCACCTCCAGC

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3801 CGCCTTCCCC TTCCGCTATC GGCTGAATT GATTGCGAGT GAGATATTAA
3851 TGCCAGCCAG CCAGACGCAG ACGCGCCGAG ACAGAACTTA ATGGGCCCGC
3901 TAACAGCGCG ATTGCTGCT GACCCAATGC GACCAGATGC TCCACGGCCA
3951 GTCGGTACCG GTCTTCATGG GAGAAAATAA TACTGTTGAT GGGTGTCTGG
4001 TCAGAGACAT CAAGAAATAA CGCCGGAAACA TTAGTGCAGG CAGCTTCCAC
4051 AGCAATGGCA TCCTGGTCAT CCAGCGGATA GTTAATGATC AGCCCACGTGA
4101 CGCGTTGCGC GAGAAGATTG TGCACCGCCG CTTTACAGGC TTCGACGCCG
4151 CTTCGTTCTA CCATCGACAC CACCACGCTG GCACCCAGTT GATCGGCCGCG
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4251 AGGTGGCAAC GCCAATCAGC AACGACTGTT TGCCCCCCAG TTGTTGTGCC
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4401 TCTGATAAGA GACACCGGCA TACTCTGCGA CATCGTATAA CGTTACTGGT
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4651 AGAATTAATT CCCAATTCCA GGCATCAAAT AAAACGAAAG GCTCAGTCGA
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4751 AGTAGGACAA ATCCGGCCCG AGCGGATTTG AACGTTGCGA AGCAACGGCC
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5051 AGGCTCACTC GAAAGACTGG GCCTTTCGTT TTATCTGTTG TTTGTCGGTG
5101 AACGCTCTCC TGAGTAGGAC AAATCCGCCG GGAGCGGATT TGAACGTTGC
5151 GAAGCAACGG CCCGGAGGGT GGCGGGCAGG ACGCCCCCA TAAACTGCCA
5201 GGAATTAATT CGAGGCATCA AATAAAACGA AAGGCTCACT CGAAAGACTC

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5251 GGCCTTCGT TTTATCTGTT GTTTGTCGGT GAACGCTCTC CTGAGTAGGA
5301 CAAATCCGCC GGGAGCGGAT TTGAACGTTG CGAACCAAACG GCCCGGAGGG
5351 TGGCGGGCAG GACGCCCGCC ATAAAATGCC AGGAATTGGG GATCGGAATT
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5451 ACTATAAGGGG AATTGTGAGC GGATAACAAT TCCCCCTCTAG AAATAATT
5501 GTTTAACTTT AAGAAGGAGA TATAcatatg gctagctcg c gagtcgacgg
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5751 CGGTGCGTAC GGTGAAAGGT CTGCGTGTGA CGGGCACCGC GAACCACCCG
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5851 GATCGACGAA ATCAAGCCGG GCGATTACGC GGTGATTCAA CGCAGGCAT
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5951 ACAACCTACA CAGTCGGCGT CCCTGGACTG GTGCGTTCT TGGAAGCACA
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6051 GGTTCTACTA CGCGAAAGTC GCCAGTGTCA CCGACGCCGG CGTGCAGCCG
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6151 GTTCGTCAGC CACGCTACTG GCCTCACCGG TCTGAACCTCA GGCCTCACGA
6201 CAAATCCTGG TGTATCCCGT TGGCAGGTCA ACACAGCTTA TACTGCGGG
6251 CAATTGGTCA CATATAACGG CAAGACGTAT AAATGTTGC AGCCCCACAC
6301 CTCCTTGGCA GGATGGGAAC CATCCAACGT TCCTGCCTTG TGGCAGCTTC
6351 AATGActccca ccaggGGAT CGGGCTGCTA ACAAGCCCG AAAGGAAGCT
6401 CACTTGGCTC CTGGCACCGC TGAGCAATAA CTAGCATAAC CCCTTGGGGC
6451 CCTCTTACCGC CTGTTGAGGG CTTTTTTGCT GAAAGGAGGA ACTATATCCG
6501 GAT

Figure 6
Hpa I ligation

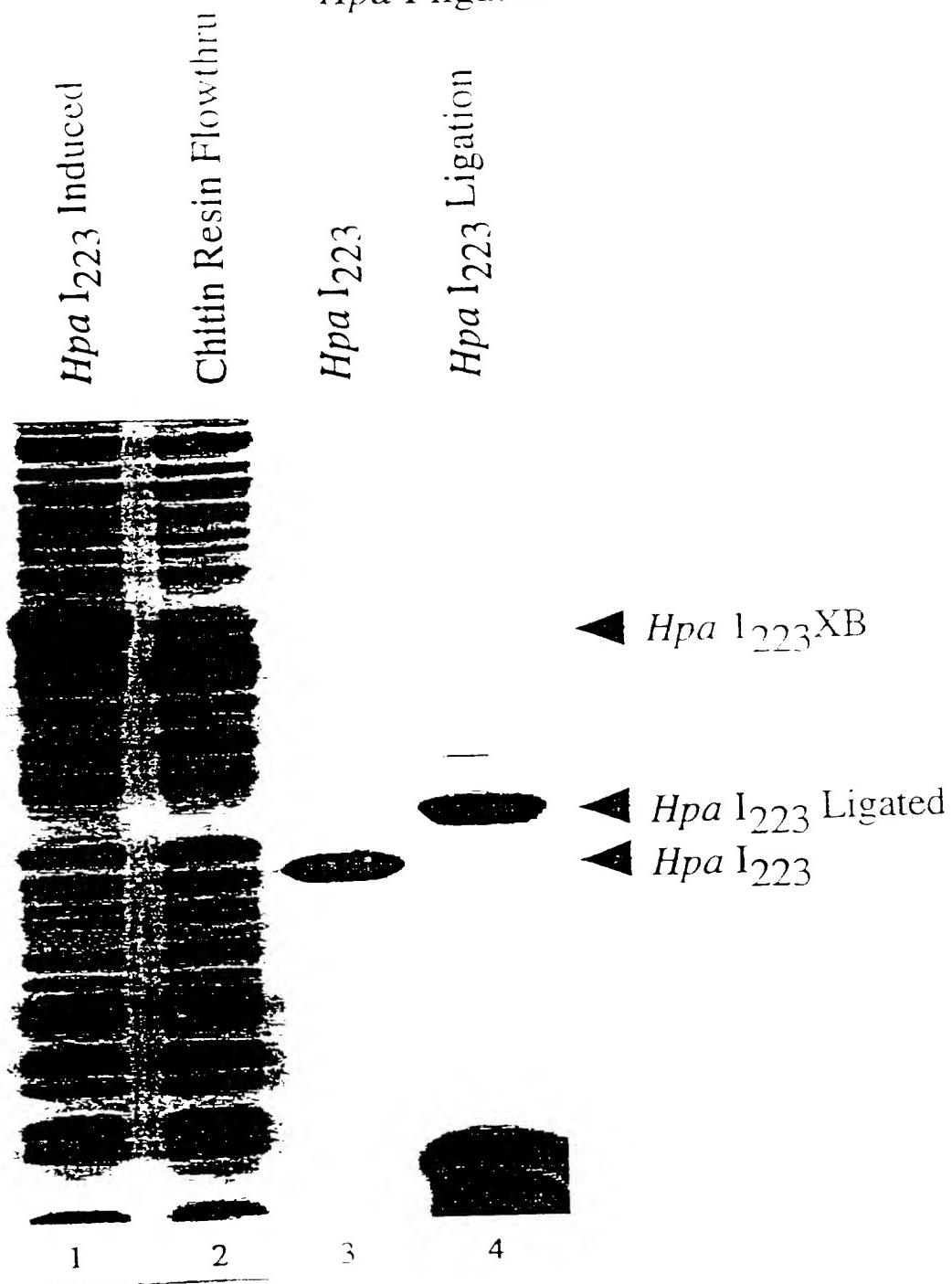


Figure 7

Western blots of Proteins Ligated to a Biotinylated Peptide

